DEVELOPMENT AND GERMINATION
OF THE AZOTOBACTER CYST

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ABSTRACT
The fine structure of *Azotobacter vinelandii* has been studied by means of electron microscopy of ultrathin sections made of the encysting and germinating cells. The organisms were fixed with KMnO₄ and embedded in epoxy resin. On an encystment medium the rod-shaped bacteria begin to assume an almost spherical form and then bark-like exine appears in 1½ to 2 days. The exine thickens and an electron permeable intine forms between it and the shrinking cell body. In 5 days the intine makes up more than half of the cyst volume and begins to show a definite two-layered structure. Meanwhile the peripheral bodies, which may be extensions of the cell membrane of the vegetative cell, disappear as the encystment progresses. The cell wall and membrane of the vegetative cell remain demonstrable as the confining structure of the shrinking central body of the mature cyst. In this central body lipoidal globules appear together with aggregations of nuclear material. Cyst germination begins with an increase in the size of the central body at the expense of the intine. The nuclear aggregations become more diffuse and the lipoidal globules disappear. The exine may be pushed outward and the bark-like fragments separate as the emerging vegetative cell develops. Invagination of the cell wall and membrane may occur at this stage leading to cell division. Empty exines remain as horseshoe-shaped structures.

INTRODUCTION
In the eubacteria there are two types of specialized resting cells, endospores and cysts. The bacterial endospore, as encountered in the genera *Bacillus* and *Clostridium*, has been extensively studied in regard to its anatomy, chemistry, and physiology. The eubacterial cyst in the genus *Azotobacter* has not been adequately characterized, although cysts of the myxobacteria and blue-green algae have been examined more thoroughly. The cyst, a thick-walled, spherical resting cell, is produced by the rounding up of a single, rod-shaped vegetative cell. Cysts have a distinctive appearance because of a contracted cytoplasm and a double-contoured cell wall. Winogradsky (1) reported that cyst formation was enhanced in some strains of azotobacter when short-chained organic compounds, such as ethanol or butanol, were supplied as the carbon source. The present, meager knowledge of azotobacter cysts stems largely from his pictures of stained preparations. A detailed cytological study by Pochon et al. (2) described the appearance of the nuclear apparatus during growth, division, encystment, and germination of the azotobacter cell as shown by the light microscope. Bisset (3) found a heat-resistant cyst formed by azotobacter, and suggested a relationship between the cyst and the bacterial endospore.

We have been concerned with the characterization of the cyst as a physiological and cytological entity. A previous paper by Socolofsky and Wyss (4) indicated that the cyst is different physiologically from the vegetative cell, based upon its ex-
treme susceptibility to lysis by the chelating agent, ethylenediaminetetraacetic acid (EDTA). Unlike endospores the resting cysts were found to be enzymatically active, to contain no dipicolinic acid, and to show only a mildly modified resistance to heat. The object of the present study is to examine the fine structure of *Azotobacter vinelandii* during the process of encystment and germination by means of electron microscopy utilizing ultrathin sections of cells.

**MATERIALS AND METHODS**

Cells of *Azotobacter vinelandii* 12837 (*Azotobacter agilis*) were grown on the surface of Burk's nitrogen-free agar (5) in Petri dishes at 33°C. Cyst formation occurred after 3 days when 0.3 per cent n-butanol was supplied as the carbon source. Since cysts germinate more rapidly on a sucrose medium, 0.5 per cent sucrose was added to the Burk's medium for germination studies.

Cells were removed from the surface of the agar plate at selected intervals during the encystment and germination processes and suspended directly in the fixative consisting of a 2 per cent solution of KMnO₄ for 6 hours at 22°C (6, 7). Following fixation each cell suspension was washed several times with distilled water before dehydrating by passage at 15 minute intervals through a graded ethyl alcohol series. Each specimen, after two 30-minute exposures of propylene oxide, was treated in a 1:3 followed by a 1:1 mixture

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**Explanation of Figures**

- *cm*, cytoplasmic membrane
- *cw*, cell wall
- *nm*, nuclear material
- *ex*, exine
- *in*, intine
- *pb*, peripheral body
- *lg*, lipoidal globule
- *db*, electron-dense body
- *cb*, central body

**FIGURES 1 TO 7**

Electron micrographs of stages in formation of cysts in members of the genus *Azotobacter*.

**FIGURE 1**

Large vegetative cells of *Azotobacter*, approximately 12 hours after transfer to an encysting medium. Discernible are the cell wall-cytoplasmic membrane complex (*cw-cm*) and nuclear material (*nm*). Note the peripheral bodies (*pb*) which appear to be located near the limiting membrane and at times give indication of attachment to it. These are observed only in young cells. X 25,000.

**FIGURE 1a**

Cross-section near the end of a cell showing distribution of the peripheral bodies throughout the entire section. X 25,000.

**FIGURE 2**

Cells which after growth on encystment medium for 24 to 36 hours have started to round up and become smaller. Peripheral bodies have disappeared and lipoidal globules (*lg*) have become prominent. X 25,000.

**FIGURE 3**

A thin section at 36 hours showing a few laminations of the exine (*ex*) deposited around the cells. X 25,000.

**FIGURE 4**

At 48 hours the exine is more prominent and electron-dense bodies (*db*) are appearing in the cytoplasm. X 25,000.
of propylene oxide: Araldite casting resin M for 1 hour. Subsequently, the specimen was subjected to treatment in a 3:1 mixture of the propylene oxide: resin for 12 hours and finally embedded in pure resin at 80°C for 24 hours. Ultrathin sections were cut on a Porter-Blum ultramicrotome with a diamond knife and were examined by means of an RCA electron microscope, model EMU-3D.

RESULTS
Cyst Formation

Cyst formation occurs from the 3rd to 5th day after growth on a suitable encysting medium. A sequence of morphological states occurring during the encystment process is presented in Figs. 1 to 7. The development of the cyst will be considered in four stages. These divisions were arbitrarily chosen and are employed to facilitate discussion of the gradual changes observed in the many pictures taken during the process.

Vegetative Cell Stage: Vegetative cells taken from a 1 day culture on an encysting medium are shown in Fig. 1. At this stage no differentiation indicative of encystment can be observed, since they appear identical with young vegetative cells growing on a non-encysting medium. The cells are typically large rods, many of which are in the process of division. A surface layer is evident and the cytoplasm is fairly homogeneous. A discrete network or band of circular-appearing structures is observed at the periphery of the cell. These bodies may be attached to the membrane and only in a polar section (Fig. 1a) is uniform distribution observed. In some sections these appear as invaginations of the cell membrane which assume the form of small tubes leading inward about 0.1 micron beneath the cell surface. Packets of material of low electron density may be discerned in the cytoplasm of ultrathin sections interspersed in a manner characteristic of nuclear material. This was confirmed using the EDTA method of Ryter and Kellenberger (8) and by examination under the light microscope of Giemsa-stained preparations.

Compaction Stage: After about 24 to 36 hours on an encystment medium the resting vegetative cells round up to an oval or spherical shape (Fig. 2); the average cell diameter is slightly smaller and the cells are much shorter than the dividing vegetative cells. The cell wall–cytoplasmic membrane complex appears much as in the younger cells, but the network of bodies on the periphery of the cell has almost disappeared. The nuclear material has become partially masked by the formation of large globular inclusions of low electron density. Examination under the light microscope of preparations which were stained with sudan black B showed these to be lipoidal globules.

Transitional Stage: In the next structural development of the encystment process, a bark-like coat or exine is deposited around the periphery of the cell. A few laminations (Fig. 3) of the structural ma-

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**Figure 5**
The exine has completely encased the cell central body (cb) and an intine (in) is developing. × 25,000.

**Figure 6**
The intine (in) has increased in size with a corresponding reduction in the size of the central body (cb). × 25,000.

**Figure 7**
Section of mature encysted cells. The thick, laminated exine (ex) and the intine (in), which at times appears to be differentiated into two layers are conspicuous. Lipoidal globules (lg) nuclear material (nm), and electron-dense bodies (db) are apparent. It is difficult to distinguish the cell wall from the cytoplasmic membrane of the central body, although a confining structure is present. × 45,000.

**Figure 7a**
Mature cyst showing decrease in size of central body as compared to Figs. 5 and 6. × 25,000.
terial of the exine may be observed in some cells at 36 hours and is generally present on the majority of them by 48 hours. During this early period of formation, the exine appears very fragile. The primordial exine does not appear to encompass the cell tightly but is observed as a loose fitting envelope enscribing the cell (Fig. 4).

Between the exine and the body of the cell lies an area that Winogradsky (1) named the intine. This layer, only slight at first, is later destined to make up more than half the volume of the cyst. The intine in the younger encysting cells appears to be nothing but an empty area at 36 hours, but by 48 hours it becomes somewhat homogeneously granular and starts to increase in size (Figs. 5 and 6).

During this period the body of the cell, which can now be termed the central body of the cyst, has not decreased appreciably in size as compared with the cells of the second stage. The cytoplasmic membrane–cell wall complex is still evident but the peripheral structures have disappeared. The lipoidal globules are prominent and the nuclear material appears more concentrated than in vegetative cells.

Cyst Maturation Stage: During the 3rd to 5th day of the encystment process, outstanding structural changes occur which may be associated with the maturation of the cyst. The mature cyst as shown in Fig. 7 has a very thick, laminated exine. The cell wall–cytoplasmic membrane component of the central body is well defined as are the lipoidal inclusions. Dispersed packets of nuclear material remain evident. However, the most striking change of this stage is the reduction in size of the central body with a corresponding increase in the size of the intine (Fig. 7a). Moreover, the intine material has increased in electron density and appears in some instances to be composed of multiple layers differing in electron density. The outer zone is more finely granular and more dense than the inner zone.

One remaining observation concerning the encystment process is the development of intensely electron-dense, small round bodies which are located randomly or in aggregates in the central body (Figs. 4 to 7).

Cyst Germination

When placed in a suitable environment, the azotobacter cysts undergo a germination process in which the exine ruptures and a large vegetative cell is liberated. On a nitrogen-free sucrose medium this process takes 6 to 12 hours.

Enlargement of Central Body: The first visible change, occurring during the 2 to 4 hour period following transfer to a germination medium, consists of the enlargement of the central body. The cyst does not enlarge during this process, but the intine becomes much less pronounced, being replaced by the increase in size of the central body (Fig. 8). The exine appears to be as thick and in-
tact as in the mature cyst, but the lipoidal inclusions are disappearing. The peripheral bodies, which were noted in the young vegetative cells of the encysting culture and which had disappeared as encystment progressed, are now discernible as shadowy outlines. The intensely electron-dense, small round bodies in the central body are disappearing at this stage.

Rupture of Exine: The next conspicuous change during the germination of cysts is a rupture of the exine (Fig. 9). At the time of rupture, the exine is more fragile and individual laminations have become apparent. The empty exines have a characteristic "horseshoe" appearance. Occasionally the young, vegetative cell begins to divide prior to rupture of the exine (Fig. 10).

The cytoplasm of the developing vegetative cell (Fig. 11) becomes more homogeneous than that encountered in the mature cyst. Lipoidal inclusions decrease both in size and number. The peripheral bodies are defined more clearly and at several points give evidence of attachment to the cytoplasmic membrane. However, the number of peripheral bodies seen in this section is great in comparison to the number of invaginations, suggesting that the bodies may be discrete structures having no direct attachment to the membrane. The cell wall and cytoplasmic membrane both appear as double-layered structures. A less electron-dense area may be seen between the wall and the membrane.

**Artificial Rupture of the Exine**

An electron micrograph of a section of a mature cyst ruptured by EDTA (4) is shown in Fig. 12. The structure of the intine has been disrupted so that it is observed in this section as an amorphous mass of viscous material still partially surrounding the central body with streams of it clinging to the exine.

Phase microscope observations indicate the viscous nature of the intine. Using phase optics it is apparent that the rupture process occurs instantaneously upon contact of the exine with EDTA. The central body appears to be forcibly ejected from the cyst during rupture of the exine and in many cases remains partially attached to the exine by the intine. In other instances the central body may be completely freed from the "horseshoe" exine structures. It is apparent that the central body is still intact and has a wall and membrane (Fig. 12a).

**DISCUSSION**

The azotobacter are excellent cytological material, as they are larger than most eubacteria. The information of a specialized resting cell offers an opportunity to study cytologically and physiologically the differentiation process at the single cell level.

The encystment process is a continuous event in which the vegetative cells, when placed on a proper medium, first gradually round up and become smaller. Later a heavy wall or exine develops, during which time the central body of the cell becomes encased in a thick, viscous intine which lies directly beneath the exine. The entire process is accomplished in 3 to 5 days.

The heavy, bark-like exine gives the cyst a distinctive appearance. The exine appears to be formed by laminations of the basic structural material. During germination the individual laminations appear to separate and become somewhat thinner, as would be expected if enzymatic degradation occurred. This is in sharp contrast to its appearance after artificial rupture by EDTA.

Preliminary studies in our laboratory have shown the exine to be composed primarily of glucose and rhamnose units; the intine appears to consist of the same basic sugar residues. It is conceivable that during the encystment process, a polysaccharide could be formed in which the basic units are polymerized to yield the definite structural form of the exine. The cell may con-
Mature cyst which has been chemically ruptured by treatment with EDTA. The exine (ex) is still thick and bark-like. Strands of the intine material (in) are seen extending from the central body (cb) to the exine. X 40,000.

Central body from EDTA-ruptured cyst showing an apparently intact cell wall (cw) and cytoplasmic membrane (cm). A disrupted area with a dense center, characteristic of EDTA-ruptured cysts, is observed. X 40,000.

Immunologic studies in our laboratory (4) have indicated that neither the capsular polysaccharide nor the exines are able to excite antibody production in rabbits. Furthermore, antibodies formed in response to injection of cysts or vegetative cells react with vegetative cells and not with cysts. The central bodies from artificially ruptured cysts may, however, be agglutinated by antibodies prepared from either cysts or vegetative cells, indicating that the antigenic determinants are exposed on removal of the exine. It would appear that the “capsule” of the cyst has undergone a sort of differentiation in which two layers have become apparent, an exine and an intine. Occasionally the intine is further differentiated into two distinct areas, yielding a capsule of greater complexity than those formed in other representatives of the eubacteria.

Aside from the development of the exine and
The most interesting aspect of encystment involves the circular-appearing structures observed at the periphery of the cell which give evidence of being attached to the cytoplasmic membrane (Fig. 12). We have not seen these structures in other organisms. Because the azotobacter have been mentioned as colorless counterparts of blue-green algae (10), it is tempting to identify these structures with chromatophores seen in photosynthetic organisms such as *Rhodospirillum rubrum* (11). However, the peripheral azotobacter bodies differ markedly from bacterial chromatophores in appearance and distribution. Our present view is that these are a network of small tubules or invaginations of the cell membrane. These structures are observed to disappear as the encystment process continues but their reappearance is one of the first changes correlated with cyst germination. These changes may parallel shifts from the lower values of oxidative metabolism of the cysts to the extremely high values associated with the vegetative cells. The QO2 values of azotobacter are among the highest recorded for any organism or tissue, ranging from 2000 to 10,000 as compared with values of 200 to 300 for *Escherichia coli*. This is surprising since the respiratory enzymes of azotobacter are associated with the cell membrane (12) and the large size of the azotobacter cells would indicate an unfavorable membrane-to-cytoplasm (surface-to-volume) ratio. On the basis of this meager evidence we suggest that the peripheral bodies may offer a means of supplementing the organized enzyme systems by extending the membrane. In this way they could represent initial attempts in the evolution of mitochondria.

Except for these invaginations of the membrane the cell envelopes yield much the same appearance in all stages of encystment and germination. Although the cell wall of the central body of the cyst appears to be intact after rupture by EDTA, we have been unable to provide these artificially ejected central bodies with an environment in which they will grow. Furthermore, examination of the supernate of EDTA-disrupted cysts reveals the presence of materials which absorb strongly in the 260 m\textmu area. This appears to indicate release of nuclear-type materials from the cell. Manometric evidence of respiratory activity of ejected central bodies can be obtained only if cells are ruptured and maintained in a solution of high osmotic pressure such as those employed in the handling of protoplasts. Therefore, although the central bodies retain their cytological identity after artificial rupture of the cyst even in the absence of osmotic stabilization, the wall and membrane must be modified in some manner not apparent in the microscopic pictures.

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